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1 Susceptibility of the C2 canine mastocytoma cell line to the
2 effects of tumor necrosis factor-related apoptosis-inducing
3 ligand (TRAIL)

4

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14 *Keywords:* C2; mast cell tumor; TRAIL; TNFRSF11B; Apoptosis

15 *Abbreviations:* TRAIL, tumor necrosis factor-related apoptosis-inducing ligand;

16 MDCK, Madin Darby canine kidney; rh, recombinant human.

17

18

Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family, which preferentially induces apoptosis in cells that have undergone malignant transformation. In humans, non-neoplastic cells are normally protected from the effects of TRAIL by expressing decoy receptors, lacking death domains. In contrast, neoplastic cells tend to downregulate their decoy receptor expression, increasing their susceptibility to the pro-apoptotic effects of TRAIL, via the functional TRAIL receptors. The aim of the current study was to investigate the effect of TRAIL on the canine C2 mastocytoma cell line to determine whether this agent might be a suitable treatment for mast cell tumors in dogs.

C2 and MDCK cells were cultured with recombinant human TRAIL. Apoptosis was assessed using a Caspase 3 & 7 chemiluminescence assay and flow cytometry following Annexin V:FITC labelling. Cell metabolism was assessed using a colorimetric MTT-based assay. C2 cells demonstrated greater sensitivity to TRAIL-induced apoptosis compared to MDCK cells by all assessment methods. The dog genome assembly was searched for orthologs of TRAIL and its receptors using published sequences from other species for reference. Although a canine ortholog for TRAIL was identified, only one TRAIL receptor ortholog (TNFRSF11B) could be found. C2, but not MDCK, cells expressed mRNA for TNFRSF11B, detected by RT-PCR. In other species, TNFRSF11B is a decoy receptor, as even though it has a death domain it is secreted due to its lack of a transmembrane domain. The effect of TRAIL on the C2 cell line suggests that this cytokine might be suitable for treatment of mast cell tumors in dogs.

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, TNFSF10, Apo2L, CD253) is a relatively newly discovered member of the TNF family. TRAIL binds to specific receptors (primarily TNFRSF10 family members) expressed on the cell surface (Wiley et al., 1995, Pitti et al., 1996). In human beings, five TRAIL receptors have been identified (LeBlanc and Ashkenazi, 2003). Two of these (TNFRSF10A & B) are functional and, upon ligation with TRAIL, induce a pro-apoptotic signal via their intracellular death domains. This occurs chiefly through the extrinsic apoptotic pathway, but with some involvement of the intrinsic pathway (Pan et al., 1997, Schneider et al., 1997, Li et al., 1998). Two further receptors (TNFRSF10C & D) are also expressed on the cell surface but, due to their lack of complete death domains, they function as decoy receptors (Sheridan et al., 1997, Pan et al., 1998). TNFRSF11B (osteoprotegerin), which can also bind to TRAIL, expresses a death domain, but due to its lack of a transmembrane domain, it functions as a secreted decoy receptor (Simonet et al., 1997).

In humans, non-neoplastic cells tend to be resistant to the effects of TRAIL, due to their expression of all five TNFRSF10 receptors, whereas neoplastic cells tend to downregulate their expression of decoy receptors, increasing their sensitivity to TRAIL-induced apoptosis via the functional receptors (LeBlanc and Ashkenazi, 2003). Following extracellular receptor binding by TRAIL, a number of proteins are involved in intracellular signaling and their interaction determines the fate of the individual cell (Fulda et al., 2002, Ricci et al., 2004, Spee et al., 2006). Soluble recombinant human (rh)TRAIL has been shown to be effective against human tumor cells of several lineages both *in vitro* and *in vivo* (Ashkenazi et al., 1999, Walczak et al., 1999) and TRAIL-based therapy has been applied *in vivo* in phase I and II human

68 clinical trials (Herbst et al., 2006, Hotte et al., 2008). Incorporation of rhTRAIL-based
69 therapies into multimodality regimens (e.g. chemotherapeutics, radiation, proteosomal
70 inhibitor combinations) often demonstrates additive or synergistic efficacy (Wen et
71 al., 2000, Belka et al., 2001).

72 In non-human, non-rodent animal species, the TRAIL: TRAIL receptor system
73 is not well-characterized. Orthologs for TRAIL have been predicted from the
74 genomes of various species including horse, dog, cat and chicken. However, there
75 appears to be considerable species variation in TNFRSF10 family members.
76 TNFRSF10A orthologs have only been verified in primates (although predicted for
77 cow and pig). TNFRSF10B orthologs have been found in the mouse as well as in
78 primates and are predicted for the cat, mouse, rat, cow and pig. In contrast, the
79 TNFRSF10C/D decoy receptors have only been identified in humans to date.
80 TNFRSF11B does appear to be conserved and has been characterized in several
81 species, probably due to its other roles in regulation of bone turnover (reviewed by
82 Roodman, 2004).

83 Mast cell tumors are the most common skin malignancy in dogs, representing
84 up to 21% of tumors at this site, but are uncommon in other species (Brodey, 1970,
85 Rothwell et al., 1987). Several breeds are over-represented including Boxers, English
86 Bulldogs, Boston terriers and Chinese Shar Peis (Peters, 1969, Patnaik et al., 1984,
87 Bostock, 1986, Rothwell et al., 1987). The median survival time post-surgery for dogs
88 with poorly differentiated mast cell tumors has been reported at as few as 13 weeks
89 (Bostock et al., 1989). One major factor that has been linked with an aggressive
90 phenotype and poor prognosis is activating mutations of the stem cell factor receptor
91 (KIT) (London et al., 1999, Ma et al., 1999, Downing et al., 2002, Zemke et al., 2002,

Webster et al., 2006), which is likely to generate a pro-survival signal, protecting neoplastic cells from apoptosis.

Surgery is the treatment of choice for canine mast cell tumors, but is a local therapy, and although many tumors demonstrate sensitivity to radiation and chemotherapeutics, these modalities tend to be best used in an adjuvant minimal residual disease setting (reviewed by London, 2003). Cases with disseminated or gross disease tend to respond less favorably to chemotherapy with short survival times, underlying the need to develop new therapies for these patients (O’Keefe et al., 1987). The aim of the current study was to investigate whether the canine C2 mastocytoma cell line, which expressed mutant KIT, was susceptible to TRAIL-mediated apoptosis as the first stage in evaluating the potential of TRAIL-based therapy for mast cell tumors in dogs.

2. Materials and Methods

2.1 Cells and cell culture

The C2 canine mastocytoma cell line (Lazarus et al., 1986) was kindly donated by Prof. B. A. Helm (University of Sheffield, UK) with permission from the originator (Prof. W Gold, University of California, USA). The MDCK cell line was obtained from the ECACC. Cells were propagated at 37 °C, 5% CO₂, in 75 cm² flasks (NUNC, Hereford, UK) in culture medium consisting of Eagle’s minimal essential medium, supplemented with 5% FCS, 1% non-essential amino acids, 50 µg/ml gentamicin (all Sigma-Aldrich, Poole, UK) and 1% L-glutamine (Invitrogen, Paisley, UK). For experiments, cells were dissociated using Accutase™ (PAA Laboratories, Hampshire, UK) and cultured in phenol red-free minimal essential medium (Invitrogen),

supplemented with 10% FCS, 1% non-essential amino acids, 1% L-glutamine and 50 µg/ml gentamicin.

2.2 Quantification of apoptosis and cell metabolism

For apoptosis assays, C2 or MDCK cells were cultured at 2×10^5 /ml in 50 µl aliquots in 96-well clear-bottomed, opaque white-walled, flat-bottomed tissue culture plates (Corning, New York, USA). For cell metabolism assays, cells were cultured at 2×10^5 /ml in 100 µl aliquots in 96-well tissue culture plates (NUNC). Cells were cultured with soluble rhTRAIL (R&D systems, Abingdon, UK) at the indicated concentrations in duplicate. Cells were cultured with staurosporine (Sigma-Aldrich) at 100 nM as a positive control apoptosis-inducing agent. Cells cultured in medium alone were used as negative controls.

Cells were incubated at 37 °C, 5% CO₂ for 8 h before measuring the level of apoptosis using the Caspase 3/7 GLO assay (Promega, Southampton, UK) by adding an equal volume of the reagent to the wells containing cells. For validation of the assay, in selected wells 50 µl medium without cells was supplemented with 1 U rhCaspase 3 (BioVision, California, USA) with or without 2mM pan-caspase inhibitor (Z-VAD-FMK, R&D systems) immediately prior to addition of the reagent. After 2 h incubation, plates were analysed using a luminometer (Spectramax M2, Molecular Devices Ltd., Wokingham, UK).

Cell metabolism was determined after 24 h culture using Cell Titer 96 Aqueous One™ (Promega). Twenty microlitres of reagent were added per well and cells incubated for a further 2 h. Absorbance values at 490nm were obtained using a plate reader (Spectramax M2, Molecular Devices Ltd., Wokingham, UK).

2.3 Flow cytometric analysis of apoptosis

C2 or MDCK cells were cultured at 2×10^6 /ml in 100 μ l aliquots in 96 well plates with medium, supplemented in selected wells with rhTRAIL (100 ng/ml) or staurosporine (100 nM). Following incubation at 37 °C, 5% CO₂ for 8 h, cells were labeled using an Annexin V:FITC antibody (Abd Serotec Ltd., Oxford, UK) and analysed by flow cytometry (FACS Aria, Becton Dickinson, Erembodegem, Belgium).

3.4 Polymerase chain reaction for detection of TNFRSF11B mRNA expression

Polymerase chain reaction was used to amplify canine TNFRSF11B from cDNA prepared from C2 cells or MDCK cells using primers based on the predicted sequence (Genbank accession # XM_539146; sense: 5'-CTAACACAGAAAGGAAA TGCAAC-3'; antisense: 5'-TCATCGTCTTCTCAATGTCTTCT-3'). Briefly, RNA was isolated from cultured cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Reverse transcription of mRNA into cDNA was performed using oligo(dT)₁₅ primer and ImProm-II reverse transcriptase (Promega). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) house-keeping gene primers (Pinelli et al., 1999) were used initially to ensure that the cDNA produced was of adequate quality for PCR analysis. Each 25 μ l reaction consisted of 1 μ l cDNA with 1x NH₄ buffer (16 mM), 1x Hi-Spec Additive, dNTP (final concentration 10 mM), magnesium chloride (final concentration 2.5 mM), 0.5 units Immolase™ DNA polymerase (all from Bioline, London, UK) and 1 μ l each sense/antisense primers (10 pmol/l final concentration). Reactions were heated to 95°C for 10 min, followed by 35 cycles of 94°C for 40 s, 55°C for 30 s and 72°C for 60 s; with a final extension step of 72°C for 7 min. PCR was performed using a G Storm thermocycler (Gene Technologies Ltd., Essex, UK) and products were separated by horizontal gel

electrophoresis using 2% agarose (Bioline) gels containing 0.5 µg/ml SafeView Nucleic acid stainTM (NBS Biologicals Huntingdon, UK). DNA was visualized under 590 nm ultra-violet light, using the ImageMaster[®] VDS Gel Documentation System (Pharmacia Biotech, Uppsala, Sweden).

3. Results

Compared to MDCK cells, C2 cells demonstrated much greater caspase 3/7 activity following exposure to soluble rhTRAIL (Figure 1). Furthermore, caspase 3/7 activity could be detected in C2 cells at much lower concentrations of rhTRAIL, compared to MDCK cells, indicating a greater sensitivity to its pro-apoptotic effects. C2 cells demonstrated a consistent decrease in metabolic activity following exposure to soluble rhTRAIL whereas the metabolic activity of the MDCK cells changed little from baseline (Figure 2).

Flow cytometric analysis demonstrated little difference in Annexin-V labelling comparing rhTRAIL-treated and untreated MDCK cells (Figure 3). In contrast, there was a large increase in the proportion of C2 cells labelled with Annexin-V following culture with rhTRAIL (Figure 3), suggesting enhanced levels of apoptosis in the treated cells.

Only one potential canine TRAIL receptor ortholog (TNFRSF11B) could be identified by screening the dog genome assembly (http://www.ensembl.org/Canis_familiaris/index.html), with no evidence for TNFRSF10 members either by homology searching (BLAST), using protein domain-prediction software (BIOMART) or a conservation of synteny-based approach. Analysis of cDNA prepared from cultured cells demonstrated that TNFRSF11B mRNA was expressed by C2 but not MDCK cells (Figure 4).

4. Discussion

This study shows that there are differences in the effect of soluble rhTRAIL on the two cell lines studied, with C2 cells demonstrating much greater sensitivity to apoptosis than the MDCK cell line. C2 was established from a spontaneously-occurring mast cell tumor-bearing mixed-breed dog which was transplanted and propagated in BALB/c nude mice (Lazarus et al., 1986). In contrast, MDCK was established from healthy adult Cocker spaniel kidney tissue (Madin et al., 1957). Thus, there might be differences in susceptibility to the effects of TRAIL that are dependent upon whether the cell has undergone spontaneous malignant transformation or *in vitro* immortalization. Interestingly, human foreskin fibroblast and human embryonic kidney cells that have been immortalized using sv40 and telomerase were not susceptible to rhTRAIL-induced apoptosis, unless additionally transformed using active ras (Nesterov et al., 2004).

Non-neoplastic human cells tend to express the full repertoire of TRAIL receptors whereas neoplastic cells tend to downregulate decoy receptor expression while continuing to express effector receptors (LeBlanc and Ashkenazi, 2003). Such differences in the receptor expression profile are likely to contribute to the cancer-specific apoptotic effect of TRAIL on human cell lines (Meng et al., 2000). The results of the current study are consistent with differential effects of TRAIL on a cell line derived from neoplastic tissue (C2) versus one derived from normal tissue (MDCK). However, further work is required with a larger number of different cell lines derived from neoplastic and non-neoplastic tissue to test this hypothesis.

There is a canine ortholog of TRAIL, located on chromosome 34 (Ensembl dog genome server: ENSCAFG000000015383). Analysis of the predicted protein

sequence and structure of human and canine TRAIL shows that there is 87% identity at the amino acid level in the extracellular region containing the TNF-like domains, which likely accounts for the cross-reactivity of rhTRAIL on canine cells. The concentrations of rhTRAIL used in the current study (0.1-1000 ng/ml) are consistent with those reported in the literature, where most susceptible cell lines show an ED₅₀ between 1-100 ng/ml.

Although a TRAIL ortholog was identified in the dog genome assembly, it was not possible to find any orthologs for the human TNFRSF10 family members. It was possible to locate orthologs of both the upstream (RHOBTB2) and downstream (CHMP7) genes in the dog genome assembly that flank TNFRSF10A-D in other species. The dog genome assembly is based on a Boxer dog, a breed that is predisposed to neoplastic disease, especially lymphoma and mast cell tumors (Bostock, 1986, Priester et al., 1973). The region between RHOBTB2 and CHMP7 in the human is of the order of 0.25 Mb, whereas the corresponding region in the canine genome is of the order of 0.06 Mb. It is possible that there are canine orthologs of TNFRSF10A-D but that this region has been deleted, either in the individual Boxer dog on which the dog genome assembly was based, or in the Boxer breed as a whole. This could be a potential explanation for the predisposition of Boxers to neoplastic disease. It is interesting to note that TNFRSF10B knock-out mice are more prone to developing neoplastic diseases (Zerafa, et al. 2005, Finnberg et al., 2008).

Although no canine orthologs of the human TNFRSF10 family members could be found in the dog genome assembly, a canine TNFRSF11B ortholog was identified. It does not seem likely that a TRAIL gene would be present in the dog genome and that rhTRAIL could induce apoptosis in C2 cells, without a corresponding effector receptor to carry out its biological functions. The demonstration of TNFRSF11B

mRNA in the C2 cell line and not in the MDCK cell line is not consistent with the increased susceptibility of C2 cells to TRAIL-mediated apoptosis since this protein functions as a decoy receptor in other species. It is possible that canine TNFRSF11B is expressed on the cell surface rather than being secreted and so functions as a death receptor, although no recognizable transmembrane domain was found through the use of protein prediction software (<http://smart.embl-heidelberg.de/>). An attempt to demonstrate binding of polyhistidine-tagged rhTRAIL to the surface of C2 cells through labelling with anti-Histidine:FITC followed by flow cytometry failed (data not shown), although this is possibly due to low affinity/avidity receptor binding.

At the higher doses of rhTRAIL used, sub-optimal effects were seen on caspase and metabolic activity in C2 cells, rather than a maximal plateau effect. In addition a marginal increase in metabolic activity was seen in the MDCK cells at the highest concentrations of rhTRAIL (Fig. 2). This might be consistent with dose-dependent variation in intracellular signaling that influences cellular outcomes. For example, activation of NF- κ B, which has been reported at higher concentrations of TRAIL, might counteract apoptosis signaling pathways (Baldwin et al., 1997, Chaudhary et al., 1997, Hu et al., 1999).

Future work is warranted to more fully characterize the role of TRAIL and its potential receptors in the dog, and the importance of apoptosis effector and modulator proteins. Evaluation of several other canine neoplastic and non-neoplastic cell lines as well as primary cells from various lineages would be necessary to demonstrate the tumor cell-specific apoptotic effects of TRAIL in dogs. Should TRAIL prove to have selective anti-cancer properties in the dog, it might be a useful therapeutic molecule in a multi-modality approach to treatment of canine cancer in the future.

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Conflict of interest statement

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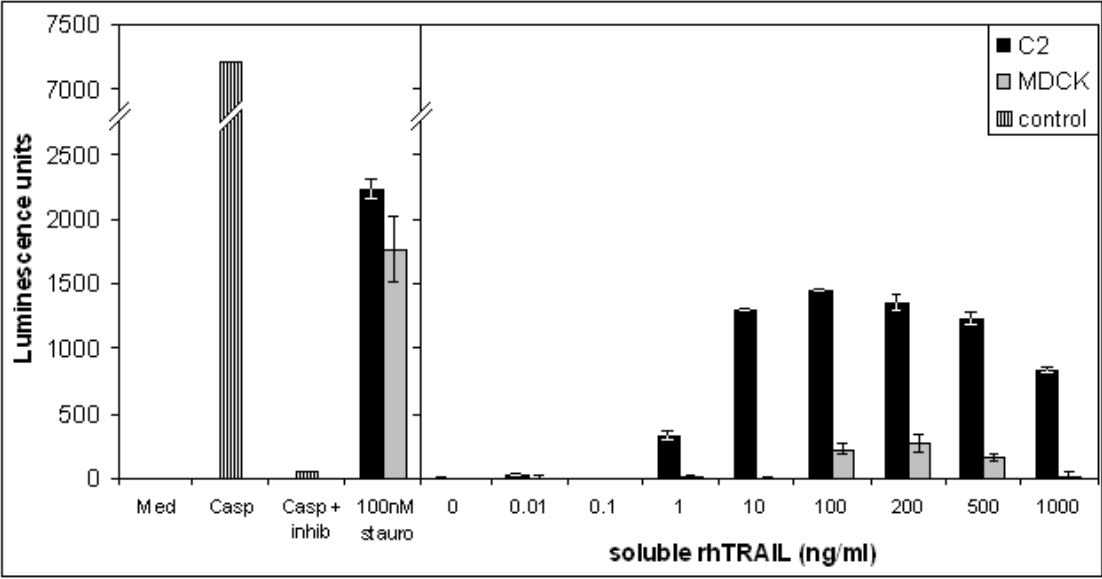
Fig. 1. Caspase 3/7 activity in C2 and MDCK cells cultured with soluble rhTRAIL. Cells were cultured for 8h and caspase activity was determined using a bioassay (Caspase 3/7 GLOTM). Results are shown as the mean luminescence value \pm SEM. The experiment was repeated twice with similar results. Controls: Med = culture medium only; Casp = culture medium supplemented with 20 U/ml rhCaspase3; Casp+inhib = culture medium supplemented with 20 U/ml rhCaspase3 and 2mM Z-VAD-FMK pancaspase inhibitor. Stauro = cells cultured in the presence of 100mM staurosporine.

Fig. 2. C2 and MDCK cell metabolism following exposure to soluble rhTRAIL. Cell metabolism was assessed using a metabolic assay (Cell Titer 96 Aqueous OneTM). Results are shown as the mean \pm SEM of the difference in absorbance values between cells cultured with rhTRAIL and cells in medium only.

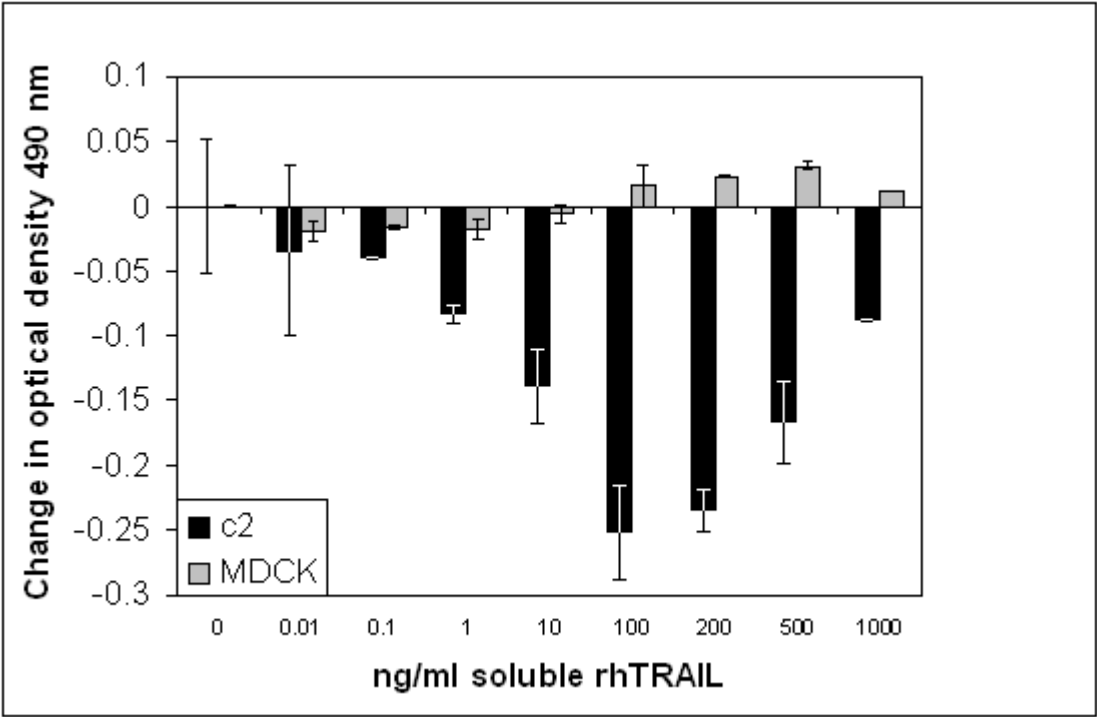
Fig. 3. Flow cytometric analysis of apoptosis in C2 and MDCK cells exposed to soluble rhTRAIL. Cells were cultured in the presence or absence of 100 ng/ml rhTRAIL for 8 h and stained with annexinV:FITC. Results are shown as fluorescence histogram overlays and mean fluorescence intensities of treated (●) and un-treated (○) cells.

Fig. 4. Assessment of canine TNFRSF11B mRNA expression by C2 and MDCK cells. cDNA prepared from C2 and MDCK cells was assessed for expression of TNFRSF11B by PCR using specific primers. Anticipated amplicon size = 432 base pairs. Ladder = 100 bp molecular weight ladder; H₂O = water negative control template.

Fig. 1.



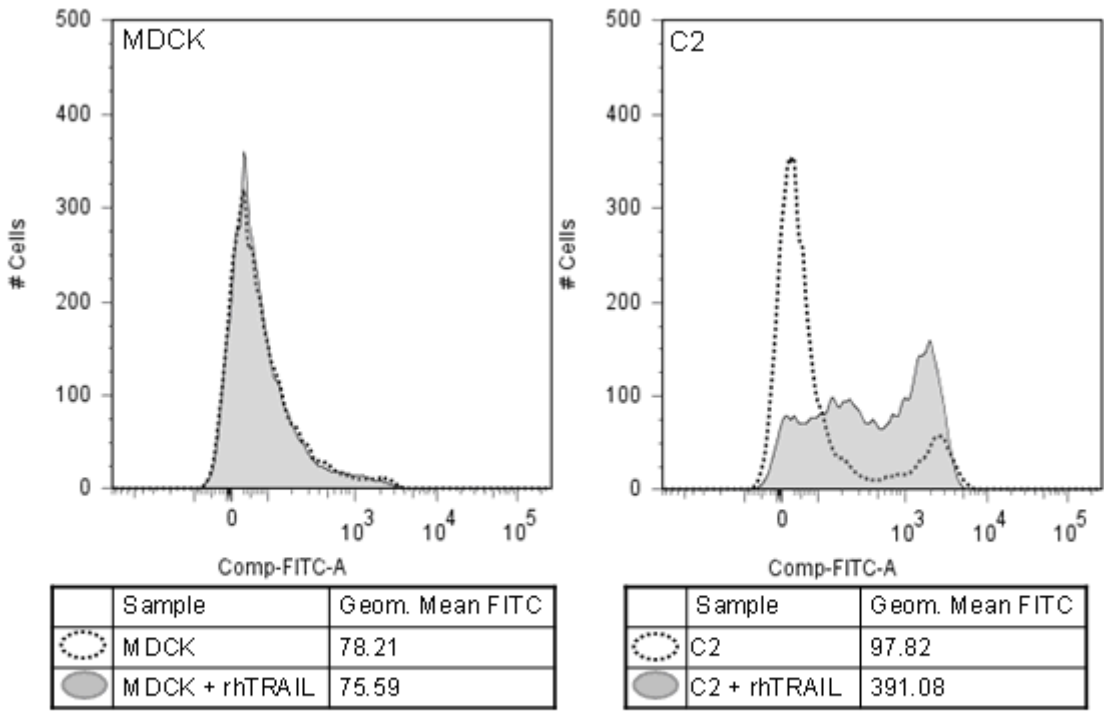
476 **Fig. 2.**



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Fig. 3.

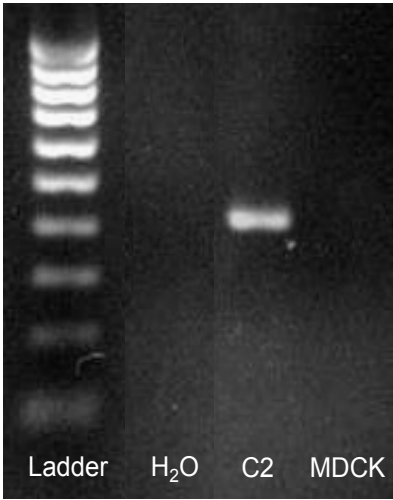


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Fig. 4.



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